STUDY ON THE RELATIVE TOXICITY OF CERTAIN ENCAPSULATED NANOPARTICLES OF PLANT ORIGIN AGAINST SELECTED MOSQUITO LARVAE

SYNOPSIS

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INTRODUCTION

Mosquitoes are important vectors carrying several pathogens causing various human dreadful diseases. Mosquitoes comprise about 3200 species belonging to 34 genera **(White *et al.*, 1987)**. *Aedes*, *Anopheles* and *Culex* are the most common genera, among the dipterans responsible for vector borne diseases to man such as vectors of dengue/dengue hemorrhagic fever (DHF), malaria and filariasis **(Bansal *et al.*, 2007)**. Mosquito borne diseases being the major health problem all over the world, are causing millions of deaths every year particularly in tropical and sub-tropical countries. Therefore, mosquitoes are being declared as "public enemy number one" **(WHO, 1996)**. *Anopheles stephensi* Linn. is the primary vector of urban malaria disseminated in India and other West Asian countries. Malaria contributes much higher than any other vector borne diseases, responsible for about 500 million infections every year, causing approximately 1.2 to 2.7 million deaths per year **(WHO, 2009)**. Control of malaria vector is, therefore, been a matter of utmost interest as previous success in the local elimination of malaria has always been accomplished wholly or in part through effective vector control. Thus, an increased understanding of vector control measures is crucial for continued progress against malaria.

Urbanization and increasing population contributes to vector proliferation by artificial larval habitats of the mosquitoes that creates increase in epidemiological trends **(Corbel *at al.* 2004).** Mosquito control programs have been suffering from failures due to increasing insecticide resistance. Synthetic insecticides have created several ecological issues due to their accumulation in the environment, effects on non-targets and development of resistance **(Nauen *et al.*, 2007).** Due to easy availablity, synthetic compounds like chlorinated hydrocarbons, pyrethroids, organophosphorous compounds, arsenical compounds etc. are frequently used in mosquito control. Control of mosquito vectors using adulticides seems not an appropriate strategy, as the adult stage occurs alongside human habitation and they can easily escape from remedial measures. Therefore, an appropriate alternative is urgently required to control them. Mosquito control at larval stage is more effective and manageable because the larval stage is restricted to aquatic habitat only **(Howard *et al.*, 2007, Shanmugasundaram *et al.*, 2001)** and larvicides can

reduce the menace of mosquito burden. Plant based compounds being environmentally benign, efficient, cost effective, may provide better efficacy to targets as an appropriate alternative for vector management.

In recent years, nanotechnology has shown a promising and considerable attention in the field of interdisciplinary research. It has a wide array of opportunities in various fields like insecticides, pharmaceuticals, electronics, and agriculture. Presently, the research regarding the mosquito management is focused on the metallic nanopesticides like platinum *(Pt)*, silver *(Ag)*, gold *(Au) and* copper *(Cu)* etc. but these metals have toxic effect on the environment, non-target organisms and human health. Therefore, the plant based nanopesticides may provide green, efficient and eco-friendly alternative for the management of mosquito vectors as they are advantageous over chemical and metallic nanoparticles **(Goodsell *et al.*, 2004)**. "Green" route for the synthesis of nanoparticles from herbal origin is of great interest due to eco-friendly and wide range of application being effectively used to control mosquitoes **(Salam *et al.*, 2012)**. Nanoencapsulation is a process in which active ingredient is confined to a cavity and surrounded by the polymer. Polymeric nanoparticles (PNPs) effectively carry the active ingredients to their target cells, tissue or organs. The encapsulated plant based nanopesticides or nanoparticles are advantageous as they may be efficiently taken by target organisms and perform may active action as compared to macro and non-encapsulated form. The nano encapsulation immensely minimizes the doses, slowly volatile and exhibits optimum effect on the target organism **(Flores *et al.*, 2011)**.

Hence, the study is planned to formulate an appropriate nano-biolarvicide against *Anopheles stephensi* to control malaria through the plant based encapsulated nanoparticles. The morpho-histochemical degenerative responses will also be studied in normal and treated *Anopheline* larvae. This study assists us in better understanding the bioactivity of the most potent nanoparticles. Thus, the proposed work is forwarding step in searching of an ecofriendly, cost-effective larvicide against *Anopheles stephensi*.

REVIEW OF LITERATURE

Intense range of literature has been studied and many workers have acquired considerable attention owing on their bioefficacy evaluation against common mosquito vectors.

I. BIOEFFICACY STUDIES:

a) MOSQUITOES AND PHYTOLARVICIDES:

Plants are eco-friendly, rich source of bioactive compounds, biodegradable, good source of useful things and far better than synthetic pesticides. Therefore, several plants have been used by various workers. **Wandscheer *et al.*, (2005)** evaluated the efficacy from the ethanolic extract of *Melia azedarach* and has studied the antifeeding and feeding deterrence activity against the dengue mosquito (*Aedes aegypti*)**. Choochote *et al.*, (2005)** performed rhizome extraction of *Curcuma aromatic* by hexane extraction and stem distillation to provide crude extracts and volatile oil and studied their repellency, adulticidal and larvicidal activity against mosquito *Aedes aegypti*. **Amer *et al.*, (2006)** evaluated 13 essential oils from 41 aromatic plants and checked their larvicidal activity against *Aedes*, *Anopheles* and *Culex*. **Mohan *et al.*, (2005)** has been used *Solanum xanthocarpum* and evaluated the larvicidal activity of roots in petroleum ether solvent against *Anopheles stephensi* and *Culex quinquefasciatus*. **Rahuman *et al.*, (2008a)** studied the larvicidal activity of crude extract of petroleum ether, ethyl acetate, acetone, methanol and hexane extracts of the leaves five plants against *Aedes aegypti* and *Culex quinquefasciatus*. **Bagavan *et al.*, (2008)** investigated that the saponin compound from the plant *Achyranthes aspera* and reported affective larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus.* **Rajwani *et al.*, (2009)** has been evaluated the larvicidal activities of three plants (*Carcia papaya L.*, *Murraya paniculata L Jack* and *Cleistanthus callinus*) against *Culex quinquefasciatus* and also studied their lethality concentration on the non-target organisms to analyze the effect of extract on the health of aquatic organisms lives in the same habitat of mosquitoes. **Govindarajan *et al.*, (2010)** reported the larvicidal activity of leaf extracts of *Ficus benghalensis* against *Anopheles stephensi* and *Culex quinquefasicatus*.

Madhu *et al.*, (2011) isolated pipyahyine from the plant, *Piper longum* and studied its activity against mosquitoes. **Aruna *et al.*, (2014)** evaluated larvicidal activity of methanolic extract of the leaves of *Citrullus lanatus* against the larvae of and *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus* **Govindarajulu *et al.*, (2015)** has been reported the crude extract of leaves *Annona reticulata* as an alternative way to eradicate the mosquito population at the larval stage.

b) MOSQUITO AND NANOPARTICLES:

Several studies have been reported by workers on the metallic and encapsulated nanoparticles to check their larvicidal activity against different species of mosquitoes.

i) Metallic nanoparticles:

Chandran *et al.*, (2006) studied the mosquitocidal and antibacterial properties of silver nanoparticles of *Aloe vera* leaf extract synthesized from silver nitrate using a cell-free aqueous leaf extract of *Aloe vera.* **Huang *et al.*, (2007)** studied the comparison between the shapes and structures of gold and silver nanoparticles formed by the leaf extract of *Cinnamomum camphora*. **Zhang *et al.*, (2010)** used chitosan/double stranded RNA nanoparticles and studied their effect by distruption of gastric caecae in *Anopheles gambiae*.

Rajakumar *et al.*, (2011) used leaf of *Eclipta prostrata* to prepare silver nanoparticles and checked their efficacy against *Culex quinquefasciatus* and *Anopheles subpietus*. **Soni *et al.*, (2012)** reported gold nanoparticles formed by entomopathogenic fungi *Aspergillus flavus* and checked their ability to the penetrating the cuticle of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*. **Sundaravadivelan *et al.*, (2013)** used silver nitrate and aqueous leaf extract of *Pedilanthus tithymaloides* to formed eco-friendly, pupicidae and bio-larvicides against *Aedes aegypti* **Banu *et al.*, (2014)** studied larvicidal activity of *Bacillus thuringiensis* nanoparticles against instar larvae of *Aedes aegypti*. **Naik *et al.*, (2014)** synthesized the nanoparticles by using plant *Pongamia pinnata* and evaluated that plant extract showed great efficiency against larvae of *Aedes aegypti* but the synthesized nanoparticles found to be the toxic to larvae. **Muthukumaran *et al.*, (2015)** reported the most stable and significant property of plant *Chomelia asiatica* based silver nanoparticles against *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus*.

ii) Encapsulated nanoparticles:

Nanomaterials are extensively growing materials to their extremely minute size and surface to volume ratio to controlling vectors which contribute to both physical and chemical differences in their properties. Among different nanoparticles, nanoencapsulation are commercialized due to their wide ranging of applications from pesticides, biosensor, water purification, agriculture, insecticides and biomedical**.** Release mechanism of polymeric nanoparticles includes dissolution, diffusion, biodegradation and osmotic pressure with specific pH **Vidyalakshmi *et al.*, (2009). Paula *et al.*, (2010)** studied the encapsulation of *Lippia sidoides* essential oil with chitosan (CH) and angico gum **(**AG**)** to studied invitro and invivo controlled release, according to their size distribution,polymer ratio and thermal stability.

Forim *et al.*, (2012) used poly-(€-caprolactone) polymer to encapsulate the extracts of neem and prepared the nanoformulations showed high entrapment efficiency when compared its UV stability with its commercial products. **Pradhan *et al.*, (2013)** encapsulated organophosphate acephate in polyethylene glycol and checked the in-vitro bioassay against an insect (*Spodoptera litura*) and tea red spider mites (*Oligonychus coffeae*). **Kah *et al.*, (2014)** reported that natural polymer nanoparticles appear to be the most promising for the active release of active ingredients (AI) due to their bioavailability, durability and would receive the more attention in future. **Bhan *et al.*, (2015)** studied the thermosensitization effect of encapsulated nanosynthetic (Temphos) and phytopesticide (*Cuscuta reflexa*) combination at different temperatures and evaluated against larvae of *Aedes aegypti* and *Culex quinquefasciatus*. **Zorzi *et al.*, (2015)** reviewed the different nanotechnologies approaches with special reference to the different plant extracts.

c) MORPHO-HISTOCHEMICAL STUDIES:

a. MORPHOLOGICAL STUDIES:

Morpho-histochemical measures in mid gut epithelium, including cell vacuolization and rupture of the epithelial walls, microvillus damage and the epithelial cell contents passing into the mid gut lumen of mosquito larvae is of great interest. Many workers reported the morphological changes in

different mosquito species at different life stages. **Khater and Shalaby *et al.*, (2008)** observed some abnormalties in *Culex pipens,* such as twisted larva, turned abdomen and changed in the pigmentation of body after the exposure of commercially available plant oils. **Arivoli *et al.*, (2011)** observed the less scleartization in the pupae cuticle, adults are unable to shed complete exuvia from their skin and inhibition of pupal-adult enclosion in the larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*.

Kumar *et al.*, (2011) reported the morphological alteration in anal gills against the larvae of *Aedes aegypti*. **Saranya *et al.*, (2013)** studied the leaf extract of *Spathodea campanulata* affect on *Aedes aegypti* and observed visible morphological deformities such as some pupal melanization (brown colour), dwarfed pupae with retarted abdomen and dechitinized larvae. **Pravin *et al.*, (2015)** treated the eggs with three different plant extracts and studied the larvae under the microscope due to swelling followed with desiccation and shrinkage. **Velu *et al.*, (2014)** reported the ruptured lumen, bursted intestinal epithelial cells and some morphological changes in the larvae of *Aedes aegypti*

b. HISTOCHEMICAL STUDIES:

As the histochemical studies being the relevant work towards the insect management and many workers has been conducted a relevant work. **King *et al.*, (1958)** evaluated the effect of tartrate on the prostatic acid phosphatases and suggested that this compound has great tendency to inhibition the activity of phosphatase in the application field if prostatic cancer. **Hussein *et al.*, (1990)** studied the activites of localization enzymes with some conventional procedure and the main activity was found in the enzyme of cholinesterase in thoracic and abdominal ganglia region of *Culex pipens.* **Corena *et al.*, (2004)** has studied the distribution of carbonic anhydrase (CA) in the midgut of *Aedes aegypti* and reported that inhibition of CA is directly related to lethal consequences for the tested species. **Zayeed *et al.*, (2009)** evaluated the separation of midgut epithelial cells from the basement membrane, destruction in microvilli and irregular appearance of columnar cells found in treated larvae of *Culex pipens*. **Fallatah *et al.*, (2010)** reported that *Trigonella foenumgraceum* extract affected the tissues of nerve ganglia, muscles, hind gut and mid gut of the larvae *Culex quinquefasciatus.*

Martins *et al.*, (2011) has studied the comparsion of mosquitoes over the fat body and reported that these bodies have a significant structural-functional relationship with an organ. **Perumalsamy *et al.*, (2013)** treated larvae with pellitoriene compound and observed the undistinguished enlarge portions of gastric caeca, alteration in thorax, midgut and found some debris in the haemolymph of anal gills in third instar larvae of *Aedes aegypti*

The development of plant based nanoparticles or nanopesticides need to be further research. Thus, sincere efforts have to unravel the possible mechanism leading to the mortality of mosquito larvae to develop encapsulated plant based nanopesticides. After reviewing the relevant literature it was observed that no proper attention has been maintained on the mosquito larvae by the application of encapsulated phyto-extract with morpho-histochemical studies. Therefore, plant based nanoparticles with special reference to polymer encapsulation method should be develop for the control of mosquito menance.

OBJECTIVES

The main objective of the proposed work is the control of *Anopheles stephensi* borne diseases by the management of their larvae through the application of eco-friendly and cost effective phyto-nanolarvicides and their morpho-histochemical impacts against the target organism. The proposed objective will be achieved by adopting the following steps:-

- Collection and extraction of fruits, leaves and roots of *Citrullus colocynthis* using petroleum ether, hexane and methanol subsequently and the respective crude extracts will be subjected to evaluate their larvicidal efficacy against *Anopheles stephensi* to find out the most potent extract.
- Chromatographic separation, purification and characterization of different fractions present in the most potent crude extract and screening their larvicidal bioefficacy against the target organisms to find out the most effective fraction.
- Preparation of phyto-nanoparticles from the most potent fraction by encapsulation with an appropriate polymer through melt-dispersion method and the prepared nanoparticles will be subjected for their larvicidal bioefficacy evaluation against target organism.
- Assessment of the morpho-histochemical impacts of the encapsulated nanoparticles to study the affected area of the gut in treated mosquito larvae.
- Assessment of the phyto-encapsulated nanoparticles against selected aquatic non-target organisms to establish the eco-friendly nature of nanoparticles for their field application.

MATERIALS AND METHODS

I.MATERIALS

a) TARGET ORGANISM:

***Anopheles stephensi* (Liston, 1901) Larvae:**

The larvae of *Anopheles stephensi* belongs to the Family: Culicidae, Order: Diptera and Sub-order: Nematocera. There are 3,357 species of culicidae are currently recognized worldwide (**Harbach 2014**). *Anopheles stephensi* being the vector of malaria is the important species threatening the society.

Identifying features:

Larvae of *Anopheles stephensi* exists in fresh water habitats such as, ponds, streams, marshes, swamps, and other sources of fresh water. Absence of air tube (Siphon) in *Anopheles* larvae make them lays parallel to the water surface. Body differentiated into three sections: head, thorax and abdomen. Adults can be distinguished from the other mosquitoes by the presence of discrete blocks of black and white scales on the wings and by the palps which are long as proboscis.

b) PLANT SELECTED:

i) *Citrullus colocynthis* (Family: Cucurbitaceae):

The plant *Citrullus coloncynthis* is selected for proposed work on the basis of its larvicidal background of leaves against mosquito, *Culex quinquefasciatus* **(Gurrudeeban, Satyavani and Ramanathan *et al.*, 2010).** The plant is native to the Mediterranean basin and Asia especially Turkey, Nubia, India and commonly known as Bitter apple, Colocynth and Desert gourd, mainly grows in sandy and arid soil. Leaves of the plant are very similar to watermelon with palmate and an angular with three to seven divided lobes. Leaves are variable in sizes from 3.8 to 6.3 cm in length and 2.5 cm in width and show pale green colour above and ashy colour beneath with deltoid margins. The roots are fleshy, large and perennial. Each plant produces 15 to 30 smooth, spheric fruits with 5 to 10 cm diameter and extremely bitter in taste. The mesocarp is filled with a soft, dry and spongy white pulp, in which the seeds are embedded and each of the carpels bears six seeds.

The leaves, seeds, fruits and roots are used to treat diabetes**,** cancer, to initiate hair growth and show antibacterial activity**.**

II. METHODS

a) REARING AND CULTURE OF MOSQUITOES:

The larvae of *Anopheles stephensi* will be collected from natural oviposition sites of the city Agra with standard dipping procedure. Identification of the larvae will be conducted by using the key **(Tyagi *et al.*, 2015)**. The Larvae will be maintained at 27±2^{o}C and 75-85% relative humidity under a photoperiod of 14:10h (light/dark) in the laboratory and will be reared in water and feed on fine powdered dog biscuits and yeast extract in ratio 3:2. Pupae will transfer from the trays to a cup containing tap water then placed into screened cages of 23×23×32 cm of diameter. The adults will be reared in the glass cages of 30×30×30 cm dimension. The adult's colony will be provided with 10% sucrose solution multivitamin syrup with periodically blood feed on shaving rats. After 3 days, ovitrap will be kept inside the cages and the eggs will be collected and transfer to the enameled trays at the same conditions. The third instars larvae will be used in bioassay tests.

b) BIOEFFICACY OF PLANT EXTRACTS

i) Extraction & preparation of test concentrations:

The plant selected will be collected from the adjacent area of Agra and the different parts of the plant like fruit, leaves and roots will be separated and washed in running tap water and subjected to shade dried. The dried plant material will be crushed and grind to make course powder. The weighed powdered material will be subjected to Soxhlet's apparatus for extraction in petroleum ether, hexane and methanol subsequently for 72 hours. Extracts will be separated from the solvent by vacuum rotary evaporator to get pure residue and finally weighed and kept in refrigerator for further use. The crude extracts of the plant will be dissolved in ethanol or acetone to get stock solutions of the desired strength and different ranges of concentrations will be prepared for each extract by diluting the stock solution in 500 ml capacity of glass beakers containing 249 ml of tap water and 1 ml of test concentration.

ii) Bioassay:

The experiments will be set according to the standard procedures **(WHO, 2005)**. Twenty, 3rd instar lab acclimatized larvae of *Anopheles stephensi* will be exposed to the desired series of different concentrations. The experiments will be conducted in triplicates along with control. Mortality count will be made after 24 and 48 hours of post treatment. Bioassay experiments showing more than 20% mortality in Control will be discarded and repeated. If the control mortality ranges between 5-20% will be corrected by using Abbott's formula **(Abbott, 1925).** The average mortality data will be subjected to probit analysis **(Finney, 1971)** to calculate LC_{50} and LC_{90} along with other statistical analysis.

c) PHYTOCHEMICAL ANALYSIS OF THE MOST POTENT CRUDE EXTRACT:

The extracts will be tested for the presence of active principle such as phytosterols, tannins, flavonoids, saponins, alkaloids, glycoside, triterpenoids and proteins. Standard procedures (Coloumn chromatography, TLC, HPLC,) will be used for separation of the different components present in the most potent extract.

a) Column chromatography of most potent extract:

The column chromatography will be conducted to isolate different components from the most potent extract. The extract will be treated with silica gel as stationary phase and combinations of different desired solvents as mobile phase for the column chromatography. All fractions will be eluted with silica gel G (80-120 mesh size), as a stationary phase and graded mixture of different desired solvents as a mobile phase. The all recovered fraction will be subjected to screen their mosquito larvicidal efficacy.

b) Qualitative analysis:

The most potent fraction will be subjected for their qualitative test and functional group such as phytosterols, tannins, flavonoids, saponins, alkaloids, glycoside, triterpenoids and proteins by following standard procedures **(Debela, 2002)**.

d) BIOEFFICACY OF ENCAPSULATED NANOPARTICLES:

i) Encapsulation:

The most potent fraction of larvicidal nature will be mixed with melted appropriate polymer and stirred lightly with a glass rod by melt dispersion method **(Peng *et al.*,, 2008)** to ensure even distribution of the mixture. The mixture is allow to crystallize at room temperature 25^{o} C, then sieve it out (200 mesh size). The sieved out nanoencapsulated particles will be kept in air tight glass containers for further use. The size and shape of these polymeric based encapsulated nanoparticles will be characterized by using Transmission electron microscopy and Nano-Zeta potential analyser.

iii) Bioassay of encapsulated nanoparticles:

The above encapsulated nanoparticles will be used to evaluate their larvicidal efficacy against the target organism as per above standard WHO procedure **(WHO 2005)**.

e) MORPHO-HISTOCHEMICAL STUDIES:

Gut is the most affected area in the insects. So the study is planned to check the histochemical changes that will be occurred in the tissues at the level of protein, as it is the important component present in larvae. Amount of protein storage accumulate at high levels during larval development. During metamorphosis they are degraded, supplying energy and amino acids for the completion of adult development.

i) Morphological study:

Treated larvae will be studied for morphological alterations under the light microscope. For the observation, larvae would be mounted on the microscopic slide with Hoyer's medium. Morphological changes if any in body segments including head, abdomen, thorax and anal gill would be observed and compared with those of control **(Kumar *et al.*,, 2011).**

ii) Histochemical analysis: For the localization of total proteins, mercuric bromophenol blue (MBB) method will be employed. **Stain Preparation**: 10 gm of mercuric chloride will be dissolved

in 100 ml of 90% alcohol. To this 100 mg of bromophenol blue to be added. This will be filtered and store.

Staining procedure

The gut tissues of third instar treated and untreated larvae will be fixed in bouins solution for 24 hours, following dehydration in a graded ethanol series (30%-100%). After dehydration, the tissues will be embedded in paraffin wax at 56-58^{o}C. The blocks are prepared for the treated parts of the gut and sections will cut with a rotatory microtome (5µ-10µ). Deparaffinized the sections and brought down to water through descending series of ethyl alcohol. The section will be stained in MBB for 15 minutes and following with washed in 0.5% acetic acid for 20 minutes. Further the sections will immersed in distilled water for 3 minutes and dehydrated in ethanol series (30%-100%) 2 dips each of it. Next step will be sectioned tissues in xylene (2 changes) and final is to be mounted in DPX (Di-butyl Phthalate Xylene) and observed under the stereomicroscope **(Pearse *et al.,*, 1975).**

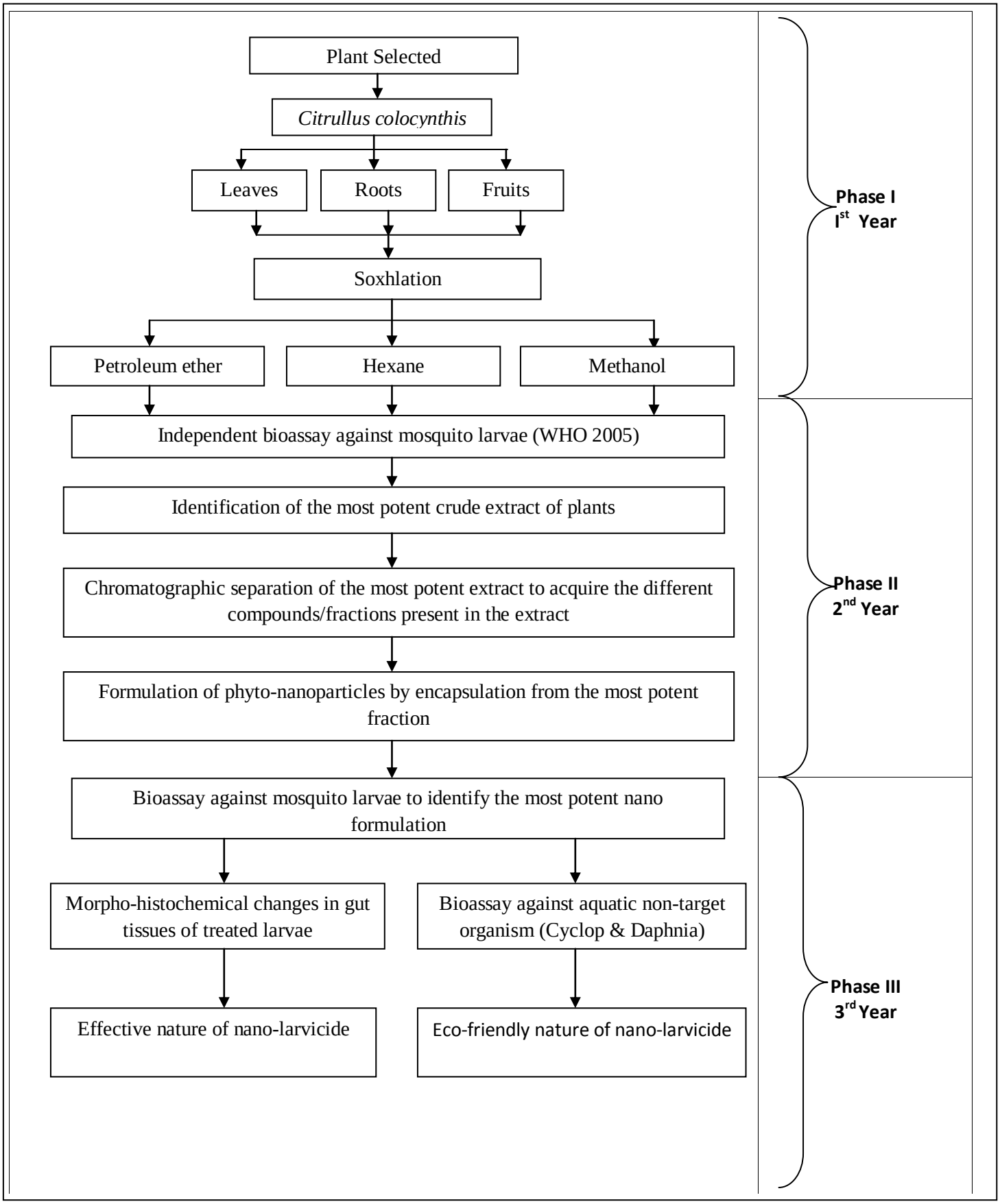
f) IMPACT ASSESSMENT OF PHYTONANOPARTICLES AGAINST NON- TARGET ORGANISMS:

The bio safety issues regarding the use of nanoencapsulation, risk assessment studies would be carried out. Various nanopreperation would be tested on selected aquatic non-target organisms such as Cyclops and *Daphnia.*

g) DATA ANALYSIS:

The observed data will be calculated by Probit analysis **(Finney, 1971)** for calculating LC_{50} and other statistical analysis of the values for all the experiments would be expressed according to the future results.

Methodology at a glance and year wise distribution of proposed work


Plant Selected
Citrullus colocynthis
Leaves
Roots
Fruits
Soxhlation
Petroleum ether
Hexane
Methanol
Independent bioassay against mosquito larvae (WHO 2005)
Identification of the most potent crude extract of plants
Chromatographic separation of the most potent extract to acquire the different compounds/fractions present in the extract
Formulation of phyto-nanoparticles by encapsulation from the most potent fraction
Bioassay against mosquito larvae to identify the most potent nano formulation
Morpho-histochemical changes in gut tissues of treated larvae
Bioassay against aquatic non-target organism (Cyclop & Daphnia)
Effective nature of nano-larvicide
Eco-friendly nature of nano-larvicide
Phase I
I st Year
Phase II
2nd Year
Phase III
3rd Year

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